약학석사학위논문

Improvement of Purification efficiency by site-directed mutagenesis in interferon beta mutein fusion antibody

돌연변이를 유발시킨 인터페론 베타 돌연변이체 융합항체의 정제효율 개선 연구

2019 년 8 월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과 분자의학 및 바이오제약 전공

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지도교수 신 영 기

이 논문을 약학석사 학위논문으로 제출함

2019년 7월

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추종완의 석사학위논문을 인준함

2019년 7월

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ABSTRACT

Improvement of Purification efficiency by site-directed mutagenesis in interferon beta mutein fusion antibody

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and College of Pharmacy, Seoul National University

Interferon β is attracting attention as an anticancer drug because it can induce the death of cancer cells. However, low stability of interferon β, induction of systemic toxicity after administration, and resistance of cancer cells are limiting the use of interferon β as an anticancer agent.

In the previous study, we have developed rhIFNβ-1a, which induces additional glycosylation of its 25 amino acid by substituting Threonine for arginine, the 27th amino acid of recombinant human interferon β 1a (rhIFNβ-1a) and named R27T. We also developed a fusion protein of R27T that was fused to the anti-ERBB-2 therapeutic antibody, Trastuzumab. In addition, Trastuzumab-C17S-R27T, in which

amino acid cysteine at position 17 of rhIFNβ-1a was replaced with serine, was developed to further improve physical properties and efficacy.

This study was conducted to compare the purification efficiency and biological properties of Trastuzumab-R27T and Trastuzumab-C17S-R27T in the purification using affinity chromatography and ion exchange chromatography. The interferon bioactivity, immunomodulatory effect, anticancer efficacy, ability to bind to HER2, and ADCC efficacy were also compared.

Trastuzumab-C17S-R27T showed improved purification efficiency and decreased aggregation in terms of physical properties when compared with Trastuzumab -R27T. It also confirmed that anti-cancer effect, interferon bioactivity and immunomodulatory efficacy were the same. In addition, the ability to bind HER2, and ADCC efficacy, was similar to Trastuzumab-R27T.

Therefore, Trastuzumab-C17S-R27T, which replaced cysteine with Serine, was found to be a more effective anticancer drug than Trastuzumab-R27T.

Key words: Interferon, Trastuzumab-C17S-R27T, Immunotherapy, Antibody, Anticancer, Fusion protein

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LIST OF ABBREVIATIONS

CD	Cluster of differentiation
DMEM	Dulbecco Modified Eagle Medium
FBS	Fetal Bovine Serum
RPMI	Roswell Park Memorial Institute
Fc	Fragment crystallizable
PBS	Phosphate-buffered saline
TEA	Triethylamine
scFv	Single chain fragment variable
HA	Hemagglutinin
TES	Tris/EDTA/Saline
IgG	Immunoglobulin gamma
ELISA	Enzyme-linked immunosorbent assay
RT	Room temperature
HRP	Horseradish peroxidase
BSA	Bovine serum albumin
FACS	Fluorescence-activated cell sorting
CDR	Complementarity-determining region
V_H	Heavy chain variable

V_L	Light chain variable
FITC	Fluorescein isothiocyanate
RIPA	Radioimmunoprecipitation assay
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
SDS	Sodium dodecyl sulfate
PMSF	Phenylmethylsulfonyl fluoride
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride
ECL	Enhanced chemiluminescence
DPBS	Dulbecco's phosphate-buffered saline
PBMC	Peripheral blood mononuclear cell
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IC_{50}	The half maximal inhibitory concentration
Fab	Fragment antigen-binding

INTRODUCTION

1. The role of interferons

Interferon is one of the major cytokines that play a role in immunity and has several potent anti-cancer effects in table 1. They are divided into type 1 (α and β interferons) and type 2 (γ or immune interferon).

Type 1 interferons, where β interferons belong, have potent antiviral activity and are produced mainly by fibroblasts and monocytes as a reaction to infection. Both α and β interferon bind to the same cellular receptor and protect uninfected cells by inducing the intracellular production of molecules that inhibit or interfere with viral RNA and DNA production. They increase the expression of MHC class I molecules leading to enhanced recognition of virally infected cells by specific cytotoxic T lymphocytes. Type 1 interferons also have antiproliferative function. β interferon reduces the relapse rate in subgroups of patients with multiple sclerosis. Interferon β has different functions, acting directly on the immune system to activate macrophage and neutrophil intracellular killing, stimulate natural killer cell function, and enhance antigen presentation by increasing MHC class II expression on antigen presenting cells. [1]

Table 1. Properties of Cytokines Under Study for Cancer Immunotherapy

Cytokine	Stimulation of T cell Immunity	Maturation of APC	Enhanced ADCC	Tumor Cell Growth Inhibition	Inhibition of Angiogenesis	Clinical Experience in Cancer	Clinical Status
IFN-α/β	+	+	+	+	+	+	Approved
IFN-γ	+	+/−	+	+	+	+/−	Approved
IL-2	+	−	+	−	−	+	Approved
IL-7	+	−	−	−	−	−	Phase I, II
IL-12	+	−	+	−	+/−	+/−	Phase I, II
IL-15	+	−	+	−	−	−	Phase I, II
IL-18	+	−	+	−	+/−	−	Phase I, II
IL-21	+	−	+	+/−	−	+/−	Phase I, II
GM-CSF	+	+	+/−	−	−	+	Approved
TNFα - 1a	+/−	+/−	−	−	+	+/−	Approved

Beyond their role in curtailing viral infection, type I IFNs play an essential part in natural cancer immunosurveillance, functioning both at the level of malignant cell precursors and through effects on the immune system.

Type I IFN signalling is also essential for the full-blown efficacy of various anticancer agents, including chemotherapeutics (such as anthracyclines), antibodies that target growth factor receptors (such as human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR)), the injection of adjuvants and oncolytic virotherapy. [2] In particular, Trastuzumab, which targets HER2, is resistant to overexpression of MUC4. This is because MUC4 prevents Trastuzumab from attaching to HER2. [3] One of the signals involved in this resistance mechanism is the homodimer of pSTAT3. [4] It is known that pSTAT3 is a homodimer that is cross regulation by pSTAT1. [5] Therefore, we believe that binding the substance that induces the pSTAT1 signal to trastuzumab would overcome resistance. The pSTAT1 signal is known to be triggered by type I and II interferons, among which antibodies have been developed that combine Trastuzumab with type I interferons with multiple anti-cancer effects.

2. Limitation of interferon β

IFN-β was more potent than IFN-α in inhibiting the cell growth of various cell lines. The spectra of the antiproliferative activity and synergistic effect of IFN-β when combined with anticancer drugs are more potent than those of IFN-α.

Combinations of IFN-β and anticancer drugs may provide a better treatment for HCC and other cancers when combinations with IFN-α are ineffective.[6]

Unfortunately, IFN therapy is associated with significant toxicity, which can be divided into the five most frequently occurring symptoms being fever (80%), myalgia (73%), headache (50%), fatigue (50%) and malaise (50%). [7]

The mechanisms of IFN-induced toxicity are unclear but are most likely multifactorial. Fatigue is the most common symptom associated with chronic use of IFN-α, occurring in more than 70% of patients. It is frequently the dose-limiting toxicity. The exact etiology of the fatigue is unclear, but there appears to be both a psychological and neuromuscular component. Chronic fatigue generally worsens with continued therapy, does not exhibit tolerance, is dose-related, and does not respond to therapy with steroids or antiinflammatory drugs. [8]

Interferon beta is also a very hydrophobic protein and tends to aggregate well. This has been shown to reduce activity and productivity. [9] In addition, there is a drawback that they have a short half-life and should be administered at frequent intervals.

Therefore, in order to reduce such Limitation, it is necessary to devise an administration method which shows a larger effect with a smaller dose. And it is had to change the physical characteristics.

3. Protein engineering for overcoming

In a previous study, introduced additional glycosylation into rhIFN-β 1a via site-directed mutagenesis. Glycoengineering had no effect on rhIFN-β ligand-receptor binding, as no loss of specific activity was observed. R27T showed improved stability and had a reduced propensity for aggregation and an increased half-life. [10]

Previously studied recombinant antibody technologies allowed the production of antibody-cytokine fusion proteins.[11] Antibody-interferon conjugates can deliver lesser amounts of interferons to specific regions of interest at a higher efficiency, thereby reducing toxicity and side effects and increasing efficacy. [12]

In this study, mutagenesis was introduced into the antibody-interferon fusion protein in the same way, referring to the results of site-directed mutagenesis in the interferon beta. [13] This is thought to contribute to the improvement of low productivity and unstable physical properties due to the inherent problem of interferon. In order to verify this, we will carry out an experiment to confirm the effect due to the antibody, and we will check the interferon signaling to confirm the effect caused by the interferon. It could be confirmed by STAT1 phosphorylation, activation of the interferon-stimulated response element (ISRE) [14], and activation of MHC class I. [15] We will also measure the ability of the fusion protein's interferon mutants to activate NK cells. [16]

MATERIALS AND METHODS

1. Construction of a mutated gene by site-directed mutagenesis

In order to establish a stable cell line and cell that transiently express mutated conjugated antibody, pCHO 1.0(Life Technologies) was used as expression vector. Among the gene constructs composed of heavy and light chains, the desired sequence was changed by site-directed mutagenesis to the heavy chain conjugated with carbiferon. This procedure was performed by transferring the heavy chain to the pOptiVEC-TOPO Circle vector. The heavy chain gene was cut with AvrII (Thermo Scientific, Pittsburgh, PA, USA) and BstZ17I(Thermo Scientific) and inserted into the pCHO 1.0 vector.

2. cell lines and culture condition

Human ovarian carcinoma (OVCAR-3), human gastric carcinoma (NCI-N87), human lung carcinoma (A549) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). iLite type I IFN assay ready cells (U937, ATCC#CRL1593.2) was purchased from the Euro Diagnostica AB (Malmo, Sweden)

OVCAR-3, NCI-N87, A549 and U937 cells were cultured in RPMI-1640 (HyClone,

Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone), 100units/mL penicillin and 100μg/mL streptomycin. They were grown at 37℃ in a humidified 5% CO_2 atmosphere.

3. Expression of protein construct in mammalian cells

The gene encoding Trastuzumab, carbiferon conjugated Trastuzumab and carbiferon-C17S conjugated Trastuzumab were transfected into CHO-S cell (Thermo Scientific) using the FreeStyle™ MAX reagent (Thermo Scientific). After OptiPRO™ SFM(Thermo Scientific) was added to FreeStyle™ MAX reagent-DNA complex, the mixture was placed on the CHO-S cell cultured in a flask, and the flask was incubated at 37℃ in a humidified 8% CO_2 atmosphere. 48 hours after transfection, proceed directly to select stable transfectants for protein expression. The cells are sorted through two selection pressures, by 10~50μg/ml of puromycin and 100~1000nM of MTX, in two-phase selection steps. The selected cells are cultured with glucose at 37℃ in a humidified 8% CO_2 atmosphere and 130RPM for 14 days to express the proteins.

4. Purification and characterization of conjugated antibody

Filtered crude harvests were purified by Protein A affinity chromatography (Mabselectsure, GE healthcare). This protein was eluted with 100mM Sodium citrate, pH 3.5. Eluted fractions were neutralized immediately with 1.5M Tris-HCl, pH8.8 and dialyzed in PBS, pH7.4. In the second purification step, ion exchange was performed using HiTrap Q FF (GE healthcare) to remove protein and DNA from the host cell. This protein was eluted with 20mM Sodium phosphate and 1M NaCl, pH 7.0. And concentrated using a 50-kDa cut-off Amicon ultra centrifugal filters Ultracel (Millipore, MS, USA). To verify protein purifies, SDS-PAGE was performed and stained with Coomassie blue. The concentration of this antibody was measured by the Cedex bio system (Roche). Finally, size exclusion chromatograph was performed using a HiLoad^{TM} 16/600 superdex^{TM} 200pg column at 1ml/min.

5. Antibodies and Western blot analysis

NCI-N87 cells were treated with rhIFNβ (0.5~50nM/ml), Trastuzumab (0.5~50nM/ml), Trastuzumab-R27T (0.5~50nM/ml), or Trastuzumab-C17S-R27T (0.5~50nM/ml), and lysed in RIPA lysis buffer (150mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50mM This-HCl [pH 7.5], 2mM EDTA) supplemented with a protease inhibitor mixture (Roche) and phosphatase inhibitor mixture (Roche). The lysed cells were incubated for 20 min on 4°C and clarified by centrifugation at 14,000 x g for 10 min. the supernatant was collected, and the protein

concentrations were determined using BCA protein assay kit (Thermo Scientific) according to the manufacturer's guide. 20 μg of protein were resolved by 10% SDS-PAGE and transferred to a polyvinyl difluoride membrane (PVDF; BioRad) the PVDF membranes were blocked in TBS-Tween20 buffer containing 5% BSA and incubated with primary antibodies at 4℃ overnight. The used primary antibodies were anti-pSTAT1 (Cell Signaling Technology) and anti-β-actin (Santa Cruz Biotechnology). And incubated with second antibodies that anti-mouse IgG-HRP (ThermoFisher Scientific) or anti-rabbit IgG-HRP (ThermoFisher Scientific) were incubated for 1 hour in room-temperature. And HRP were visualized using Clarity Western ECL substrate (Bio-Rad).

CHO-S cells' culture fluids expressing Trastuzumab-R27T, or Trastuzumab-C17S-R27T were subject to 10% SDS-PAGE, and the resolved proteins were transferred to a PVDF membrane (Bio-Rad). The PVDF membranes were blocked in TBS-Tween20 buffer containing 5% skim milk and incubated with anti-human IgG-HRP (GenScript). And HRP were visualized using Clarity Western ECL substrate (Bio-Rad).

6. Interferon β bioactivity analysis by Luciferase assay

Interferon β bioactivity were measured by Luciferases assays using iLITE type 1 IFN assay ready cells (Euro Diagnostica) and a Luciferase assay kit (Promega).

iLITE type 1 IFN assay ready cells were seeded to 96-well plates and treated with the indicated concentration of rhIFNβ (0.05~5000nM/ml), Trastuzumab (0.05~5000nM/ml), Trastuzumab-R27T (0.05~5000nM/ml), or Trastuzumab-C17S-R27T (0.05~5000nM/ml). After 18 hours, the activities of firefly luciferase were determined using a microplate reader (TECAN)

7. Immunomodulatory effect assay by FACS

The immunomodulatory effects were measured by the activation of MHC Class I in A549 cells. A549 cells were seeded to 96-well plates and treated with the indicated concentration of Trastuzumab (0.1~1000ng/ml), Trastuzumab-R27T (0.1~1000ng/ml), or Trastuzumab-C17S-R27T (0.1~1000ng/ml) for 48 hours. After treating the cells with Cell Dissociation Buffer Enzyme-Free PBS-based (Gibco), they were collected by centrifugation. Cells were diluted to a concentration of 1 x 10^7 cells/mL with FACS buffer and the expression of MHC Class I was measured by FACS analysis by BD FACS Caliber system equipped with the Cell Quest Pro software (BD Biosciences). FITC anti-human HLA-A, B, C Antibody (BioLegend) were used for detection.

8. Anti-proliferation activity

The anti-proliferation efficacy of Trastuzumab-R27T or Trastuzumab-C17S-R27T

was using viability assays and Calcein-AM assays.

Viability assays were used Water-Soluble Tetrazolium (WST) colorimetric assay (Ez-Cytox; Daeil Lab Service). NCI-N87, OVCAR-3, BT-549 and A549 cells were seeded to 96-well plates and treated with the indicated concentration of Trastuzumab (0.01~10000ng/ml), Trastuzumab-R27T (0.01~10000ng/ml), or Trastuzumab-C17S-R27T (0.01~10000ng/ml) for 72 hours. After that, 10μl of WST reagent was added to each well, the plates were incubated for 30 min, and absorbance was measured at 430 nm using a microplate reader (TECAN).

Calcein-AM assays were used Calcein-AM reagent (BioLegend). NCI-N87, OVCAR-3, BT-549 and A549 cells were seeded to 6-well plates and treated with the indicated concentration of Trastuzumab-R27T (1~10000ng/ml) or Trastuzumab-C17S-R27T (1~10000ng/ml) for 72 hours. And treat 10μl of the 1μM Calcein-AM working solution. And incubated cells for 20 minutes at 37℃ and keep protected from light. After incubation cells in culture medium for 10 minutes, and then observed using a Fluorescence Microscope (IX70; Olympus).

9. Flow Cytometry analysis

Flow cytometry analysis was used to determine the ability of the conjugated antibody to bind to HER2. NCI-N87 and BT-549 cells were harvested by Cell Dissociation Buffer Enzyme-Free PBS-based (Gibco), blocked for 1 hour at 4℃ in cold phosphate-buffered saline (PBS) containing 2% Fetal bovine serum (FBS; HyClone).

The cells were washed three times with PBS, incubated for 30 min at 4℃ with 1μg of Trastuzumab, Trastuzumab-R27T or Trastuzumab-C17S-R27T diluted by PBS. And the cells were washed three times with PBS, incubated for 30 min at 4℃ with Gaot anti Human IgG FITC (Jackson). The cells were identified by detection of fluorescent antibodies using a BD FACS Caliber system equipped with the Cell Quest Pro software (BD Biosciences).

10. Antibody-dependent cell-mediated cytotoxicity (ADCC) assay

The antibody-dependent cell-mediated cytotoxicity efficacy of Trastuzumab, Trastuzumab-R27T or Trastuzumab-C17S-R27T was using LDH assays. NCI-N87 cells were seeded 2 x 10^4 cells to 96-well plates and incubated 24 hours. Treated with the indicated concentration of Trastuzumab (0.01~10000ng/ml), Trastuzumab-R27T (0.01~10000ng/ml), or Trastuzumab-C17S-R27T (0.01~10000ng/ml). After that, 8 x 10^4 NK-92MI-CD16 cells were added and incubated 6 hours. Add 10 μl of lysis soution to the positive control 1 hour before the measurement. After that, centrifuge at 500 g and transfer the supernatant to a new plate in 50 μl increments. Add 50 ul of CytoTox 96 reagent (Promega) to the new plate, block the light and incubate for 30 minutes at room temperature. Stop solution is added in 50 μl increments and absorbance is measured at 490 nm within 1 hour using a microplate reader (TECAN).

11. Aggregation assay

An aggregation assay was performed to measure the physical properties. Dispense 2 μl of the diluted PROTEOSTAT detection reagent (Enzo). Into the bottom of each well of 96-well plate. Add 98 μl of the Trastuzumab-R27T (1mg/ml), or Trastuzumab-C17S-R27T (1mg/ml) each well. Light was blocked and incubated at room temperature for 15 minutes. Read generated signal with a fluorescence microplate reader (TECAN) using an excitation setting of about 500 nm and an emission filter of about 600 nm.

RESULTS

1. Expression and purification analysis of Trastuzumab-C17S-R27T fusion protein

The stable cell line that expresses the antibody continuously was constructed and the experiment was conducted. For comparison with the expressed Trastuzumab-C17S-R27T, Trastuzumab-R27T and Trastuzumab were also expressed and purified under the same conditions. Expression was incubated in CHO-S cell line for 10 days at 37 ° C, 5% CO2.

Expressed culture medium was assayed for antibody concentration via Cedex-bio. Each culture medium adjusted its concentration to match the conditions as closely as possible. Finally, the concentration was adjusted to 80 mg / L.

Purification was performed using an affinity chromatographic method using an AKTA instrument and a protein A bead. In most cases, Trastuzumab-C17S-R27T was determined to have high purification efficiency and purity. The purification efficiency in the affinity chromatography step was measured within a range of maximum 39% to minimum 25%.

In order to increase the purity, the first purified product was subjected to second purification through ion exchange chromatography. In the second purification,

impurities were removed considerably, and high purity could be obtained. However, the purification efficiency was relatively low as 11 ~ 17%, and the yield of the final product was 3 ~ 7%.

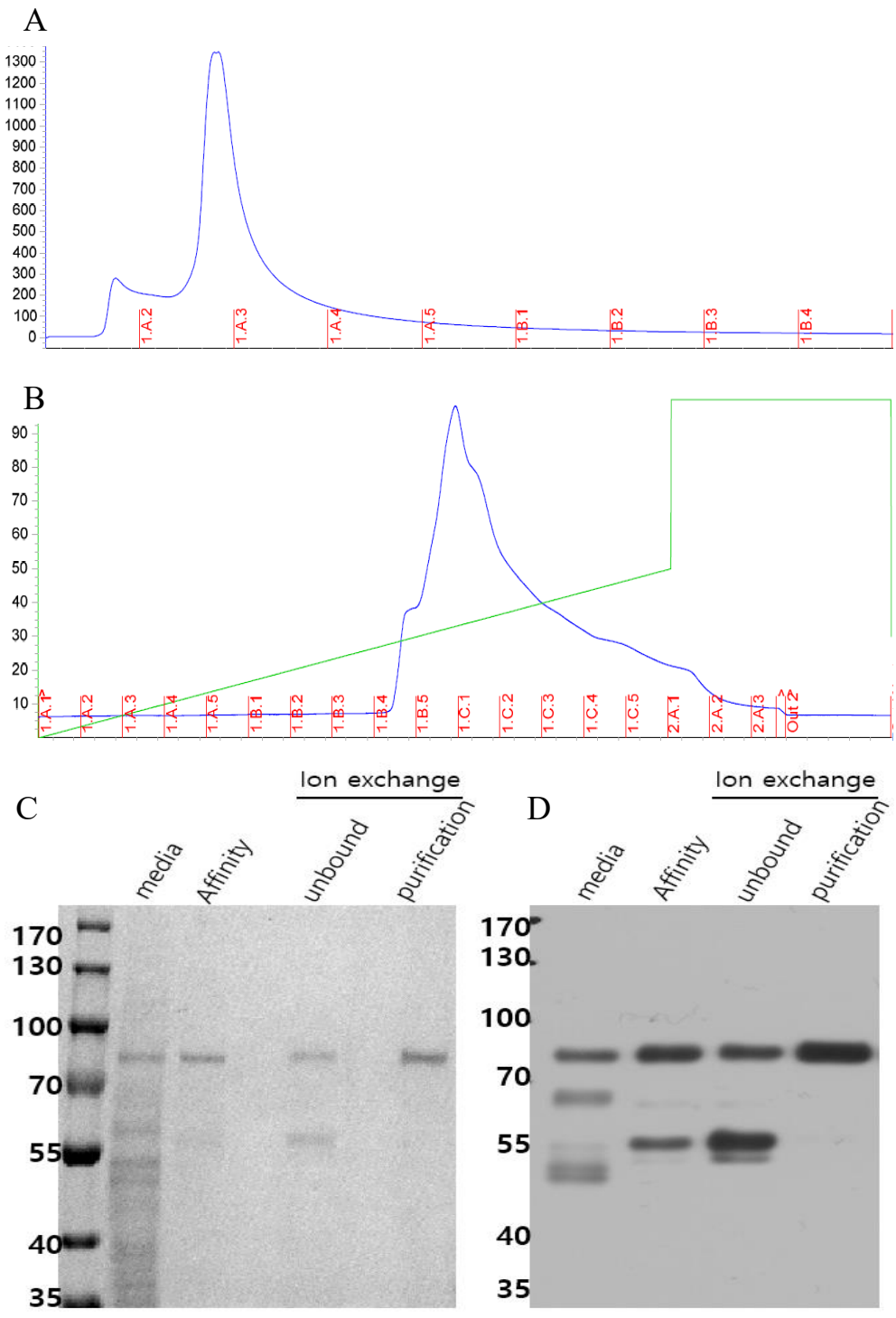
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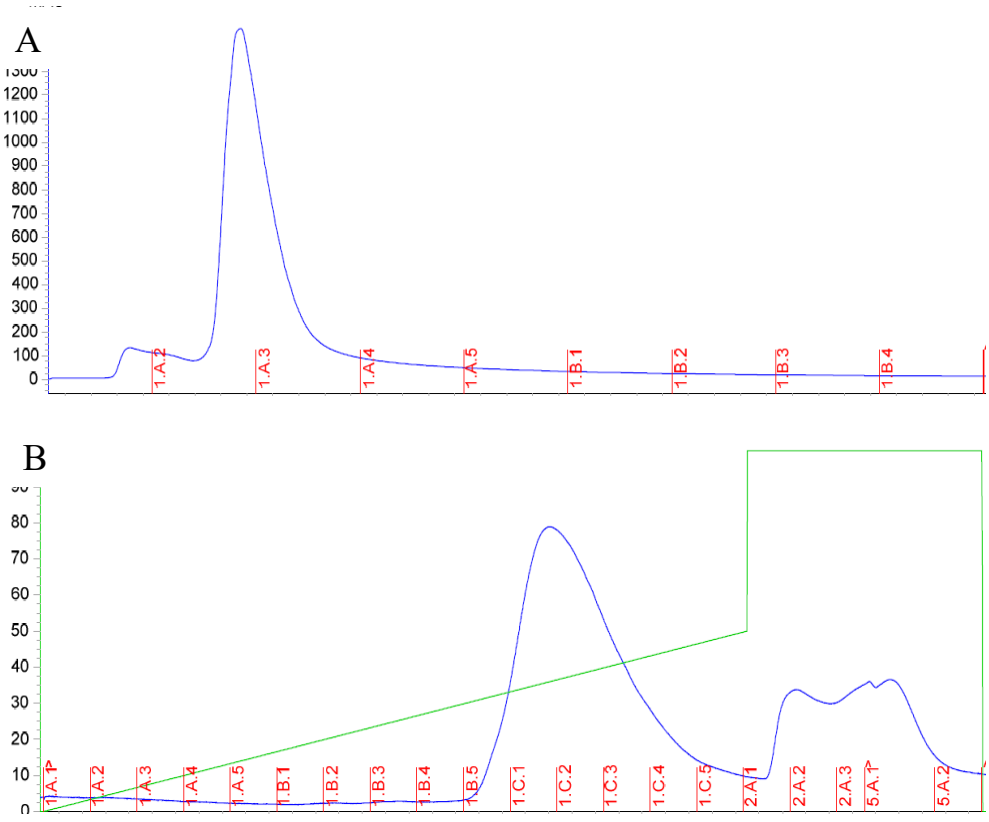
Figure 1. Purification result of Trastuzumab-R27T. (A) This is the result of affinity chromatography using protein A bead column. (B) The results of ion exchange chromatography using Q-column. Experiments were conducted by sampling the fractions corresponding to the peaks. (C) Each refined product was sampled and subjected to PAGE under reducing conditions and stained with coomassie. (D) Each refined product was sampled and subjected to PAGE under reducing conditions and Western blotting.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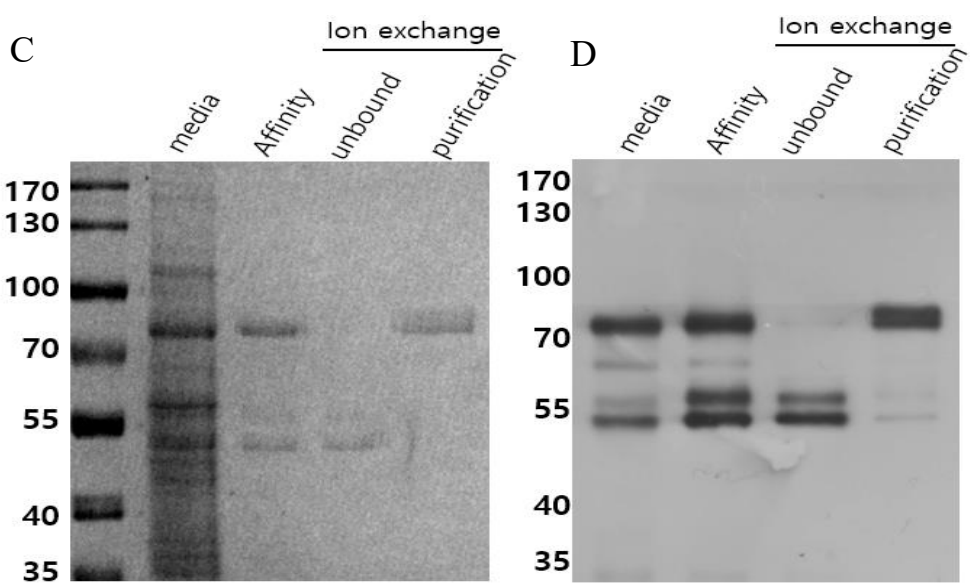
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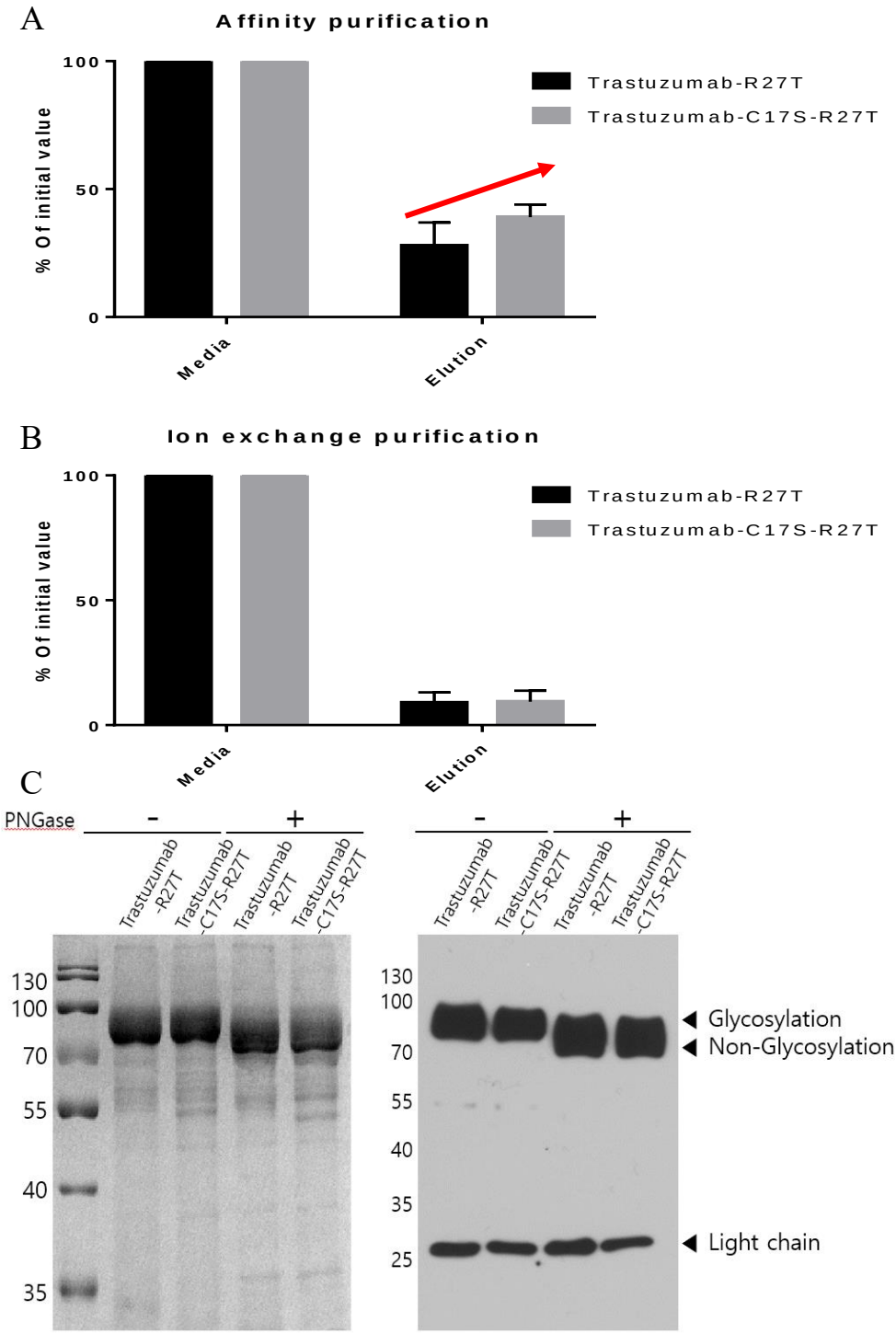
Figure 2. Purification result of Trastuzumab-C17S-R27T. (A) This is the result of affinity chromatography using protein A bead column. (B) The results of ion exchange chromatography using Q-column. Experiments were conducted by sampling the fractions corresponding to the peaks. (C) Each refined product was sampled and subjected to PAGE under reducing conditions and stained with coomassie. (D) Each refined product was sampled and subjected to PAGE under reducing conditions and Western blotting.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40
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130
100
70
55
40
35
25
Glycosylation
Non-Glycosylation
Light chain

Figure 3. Comparison of purification efficiency of Trastuzumab-R27T and Trastuzumab-C17S-R27T. (A) This is a graph comparing the yields of the affinity chromatography performed first. (B) This is a graph comparing the yields of the second-conducted ion exchange chromatography. (C) Each final purified product was treated with PNGase for 2 hours and subjected to PAGE under reducing conditions. After the progression, they were identified by coomassie blue staining and Western blotting, respectively.

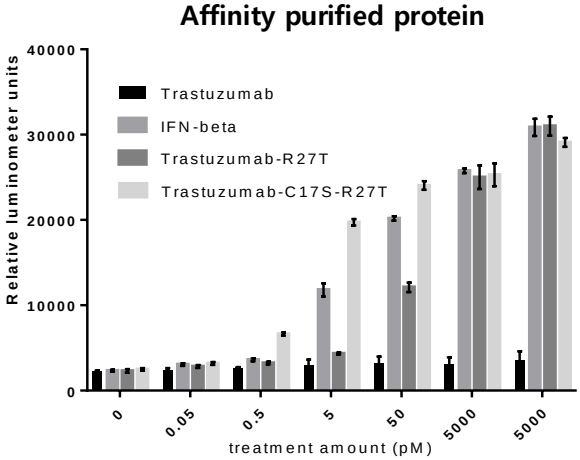
2. IFN bioactivity of Trastuzumab-C17S-R27T

To confirm the bioactivity of IFN fused to Trastuzumab-C17S-R27T, I used the following facts. IFN activity is mediated through the binding of type I IFN receptors and the subsequent phosphorylation of STATs. Therefore, I used Trastuzumab-C17S-R27T as a measure of induction of STAT1 phosphorylation in NCI-N87 cells. The results clearly showed that the signal was improved by concentration, and the activation level of Trastuzumab-C17S-R27T was superior to that of Trastuzumab-R27T.

In another way, I used the iLITE type 1 IFN assay ready cell system to confirm the bioactivity of IFN. This cell is a cell that expresses firefly luciferase under the control of IFN-responsive promotor. Cells were seeded in 96-well plates in the same number and treated with various concentrations of Trastuzumab-C17S-R27T to measure their luminescence. The results show that Trastuzumab-C17S-R27T has higher overall luminescence when compared to Trastuzumab-R27T, especially at intermediate concentrations.

Immunomodulatory effects were determined by measuring the degree of MHC Class I activation in A549 cells. FACS analysis confirmed IFN-inducible expression of MHC class I on the surface of A549 cells. The results showed that Trastuzumab-C17S-R27T showed a stronger effect at all concentrations.

A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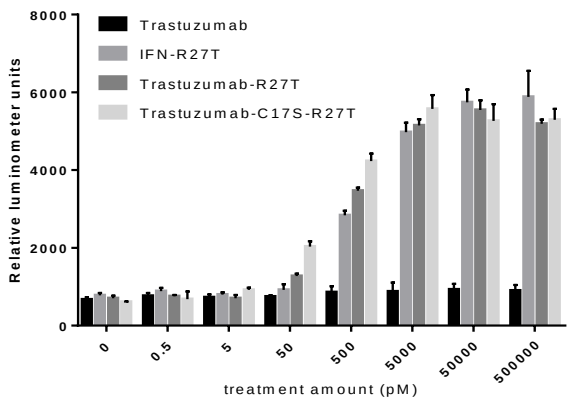
Trastuzumab
IFN-beta
Trastuzumab-R27T
Trastuzumab-C17S-R27T
0
0.05
0.5
5
50
5000
5000
treatment amount (pM)

B


Ion exchange purified protein
Relative luminometer units
8000
6000
4000
2000
0
Trastuzumab
IFN-R27T
Trastuzumab-R27T
Trastuzumab-C17S-R27T
0
0.5
5
50
500
5000
50000
500000
treatment amount (pM)

C

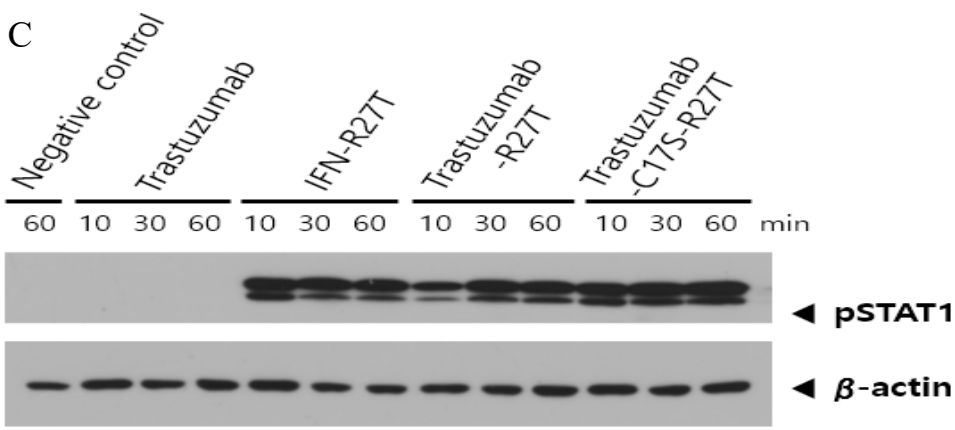
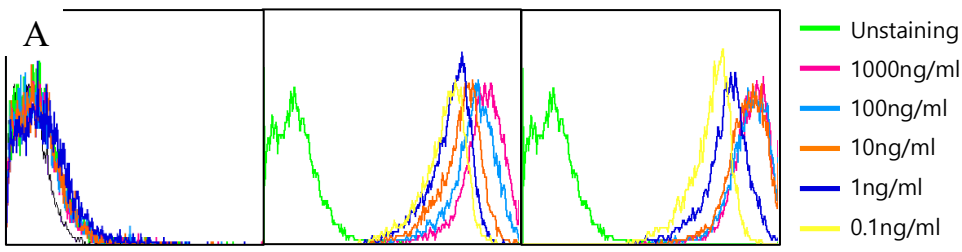
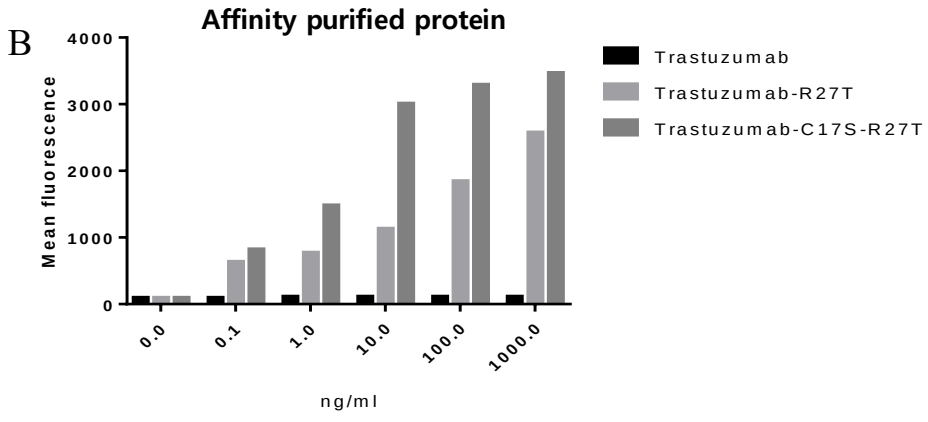
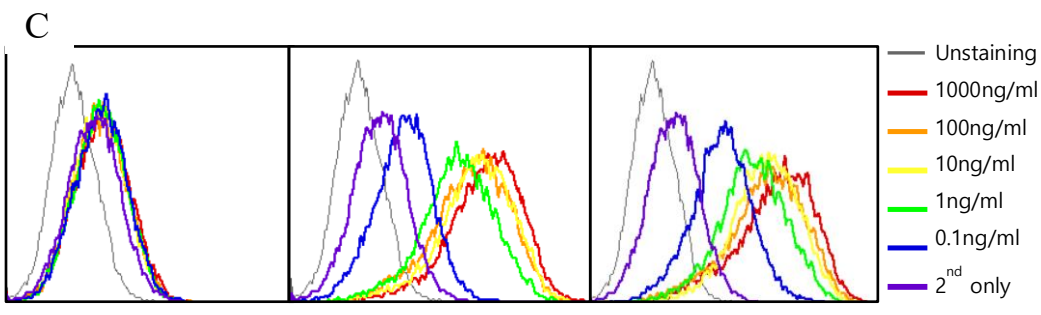

Negative control
Trastuzumab
IFN-R27T
Trastuzumab -R27T
Trastuzumab -C17S-R27T
60 10 30 60 10 30 60 10 30 60 10 30 60 min
pSTAT1
β-actin

Figure 4. IFN bioactivity of Trastuzumab-C17S-R27T. (A) The iLITE cells were seeded 1 x 10^4 in a 96-well plate and the primary purified product was treated at various concentrations as follows. After 18 hours, luciferin was treated and absorbance was measured. (B) The same procedure was followed for the secondary purification results. (C) U937 cells were seeded 5 x 10^5 in a 6-well plate and treated with secondary purification products, and after 10, 30, and 60 minutes, the cells were harvested and subjected to PAGE under reducing conditions. Western blotting was performed using anti-pSTAT1 antibody.


A
Unstaining
1000ng/ml
100ng/ml
10ng/ml
1ng/ml
0.1ng/ml


B
Affinity purified protein
Mean fluorescence
4000
3000
2000
1000
0
0.0
0.1
1.0
10.0
100.0
1000.0
ng/ml
Trastuzumab
Trastuzumab-R27T
Trastuzumab-C17S-R27T


C
Unstaining
1000ng/ml
100ng/ml
10ng/ml
1ng/ml
0.1ng/ml
2nd only

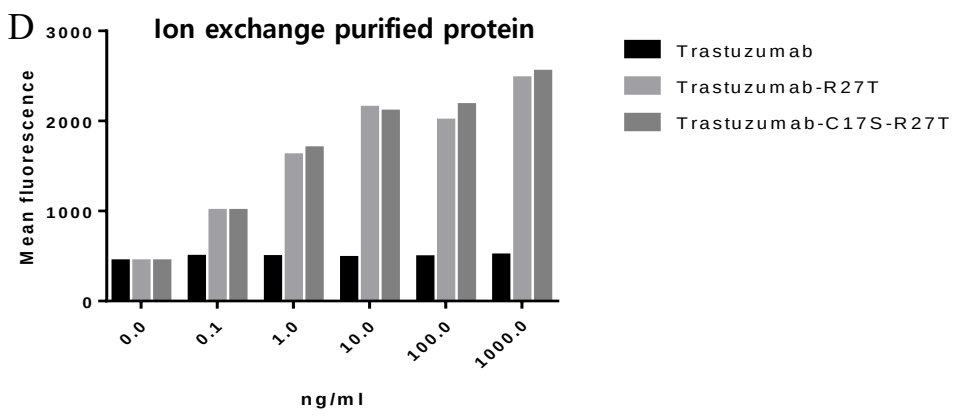

D
Ion exchange purified protein
Mean fluorescence
3000
2000
1000
0
0.0
0.1
1.0
10.0
100.0
1000.0
ng/ml
Trastuzumab
Trastuzumab-R27T
Trastuzumab-C17S-R27T

Figure 5. IFN induced immunomodulatory activity of Trastuzumab-C17S-R27T

(A) A549 cells were seeded 5 x 10^5 in a 6-well plate and treated with various concentrations of fusion proteins and analyzed by FACS using anti-human HLA A, B, C antibodies. (B) The results of the FACS analysis are shown graphically. (C) The fusion protein was subjected to secondary purification under the same conditions. (D) The results of the FACS analysis are shown graphically.

3. Targeting of ERBB-2 by Trastuzumab-C17S-R27T

FACS was used to confirm binding ability to ERBB-2. In ERBB-2 positive cells, Trastuzumab, Trastuzumab-R27T, and Trastuzumab-C17S-R27T all showed equivalent binding ability, whereas ERBB-2 negative cells did not bind to all antibodies.

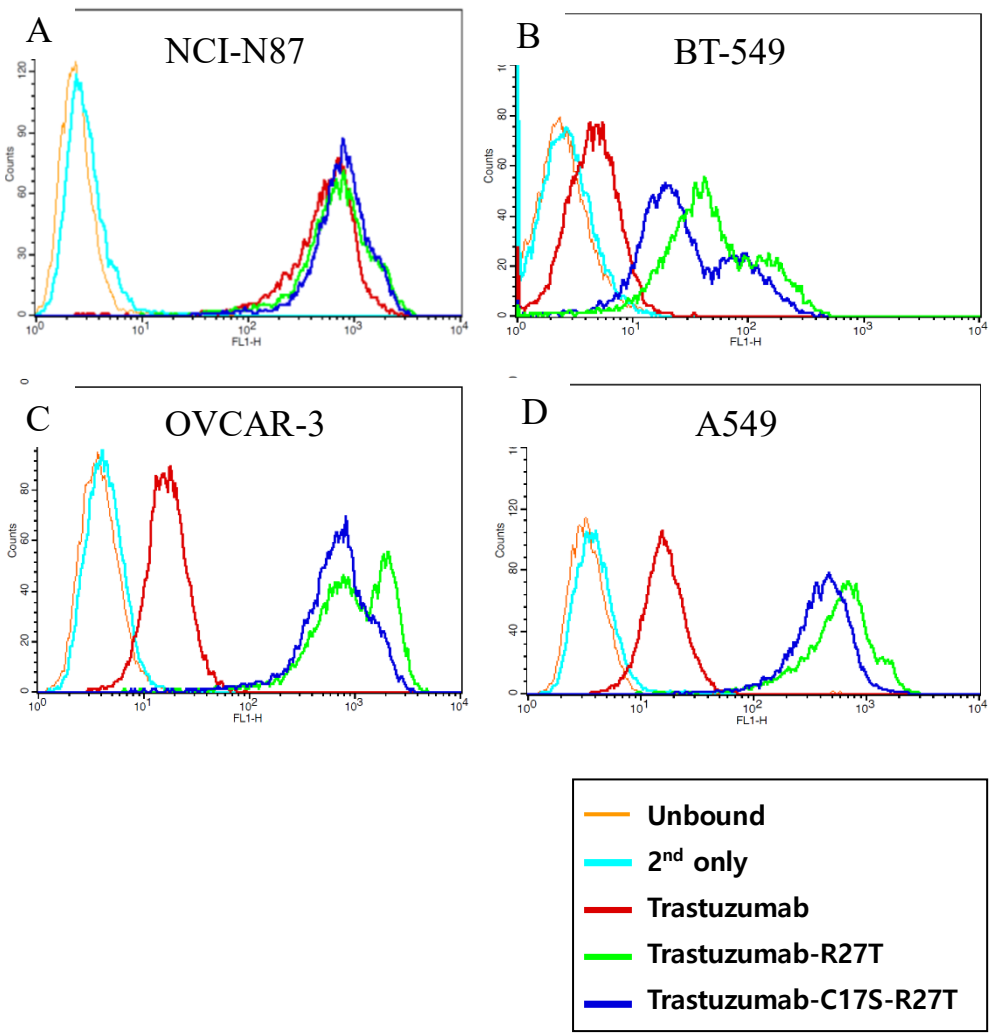

A
NCI-N87
B
BT-549
C
OVCAR-3
D
A549
Counts
FL1-H
Unbound
2nd only
Trastuzumab
Trastuzumab-R27T
Trastuzumab-C17S-R27T

Figure 6. Analysis of ERBB-2-targeting function by FACS.

(A) FACS analysis was performed using anti-human IgG antibody after treating the fusion protein with NCI-N87, HER2 positive cell, for 1 hour. (B) FACS analysis was performed on ERBB-2 negative cells BT-549 under the same conditions. (C), (D) The same assay was performed using cell lines in which HER2 was not overexpressed.

4. Anti-cancer efficacy of Trastuzumab-C17S-R27T

In this experiment, anti-cancer effect was confirmed by two methods. After 72 hours, the number of living cells was measured using WST. The results showed a boiling killing effect in NCI-N87 cells, and more efficacy in Ovcar-3 cells. In the negative control group, no killing effect was observed.

Four cell lines were treated with the same fusion protein and fluorescently imaged live cells by staining with Calcein-AM. Similar to the results of WST, NCI-N87 showed similar efficacy and Ovcar-3 showed better efficacy.

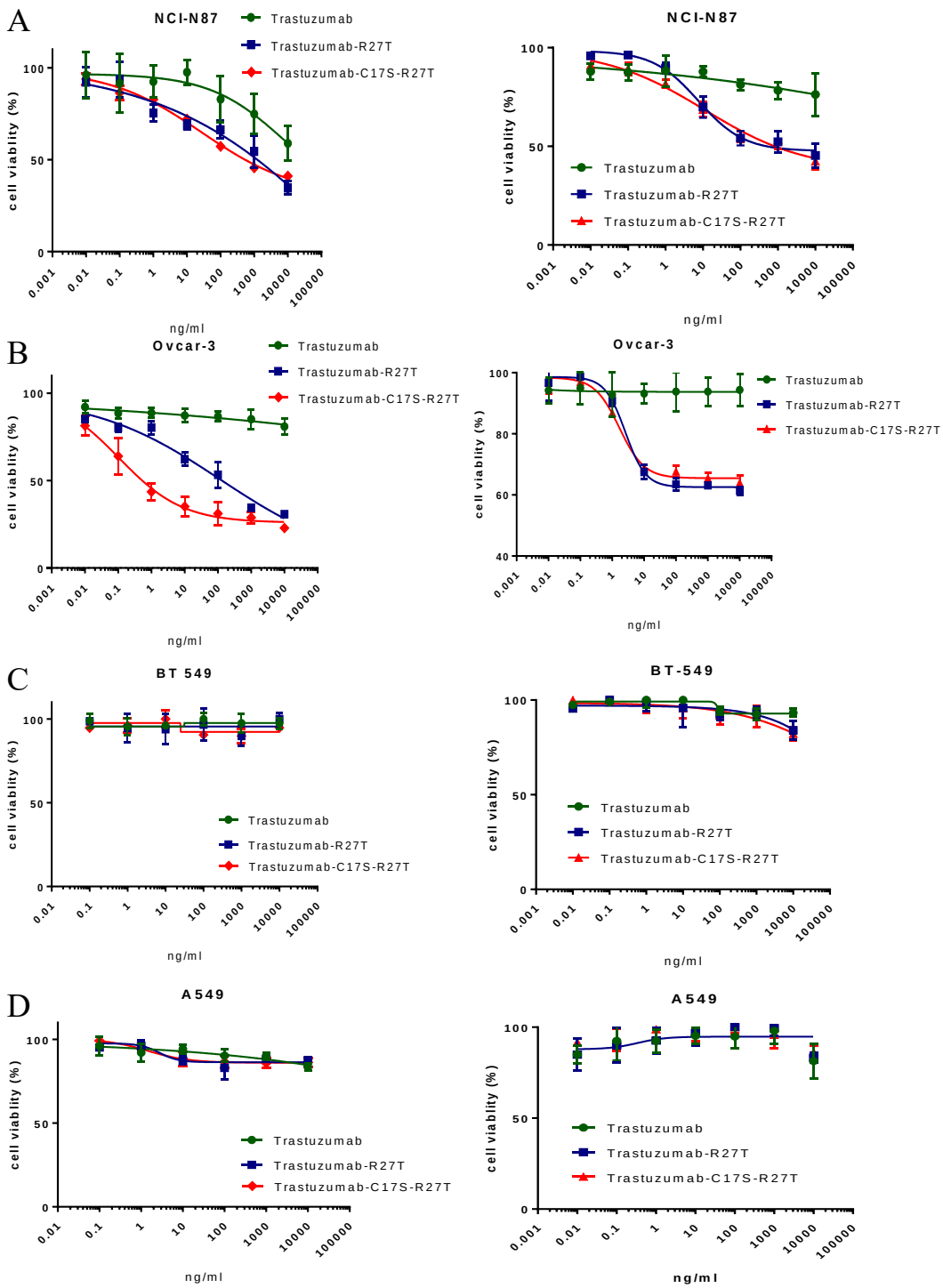

A
NCI-N87
Trastuzumab
Trastuzumab-R27T
Trastuzumab-C17S-R27T
cell viablity (%)
ng/ml
B
Ovcar-3
C
BT 549
BT-549
D
A549

Figure 7. In vitro anti-cancer efficacy by WST assay

(A) NCI-N87, ERBB-2-positive cells, was seeded 1 x 10^4 in a 96-well plate and, treated with various concentrations of fusion protein 1 day later, and WST assay was performed after 2 days. (B) The same experiment was performed on BT-549, ERBB-2- negative cells, under the same conditions. (C) and (D), The same experiment was performed on BT-549 and A549 as negative controls.

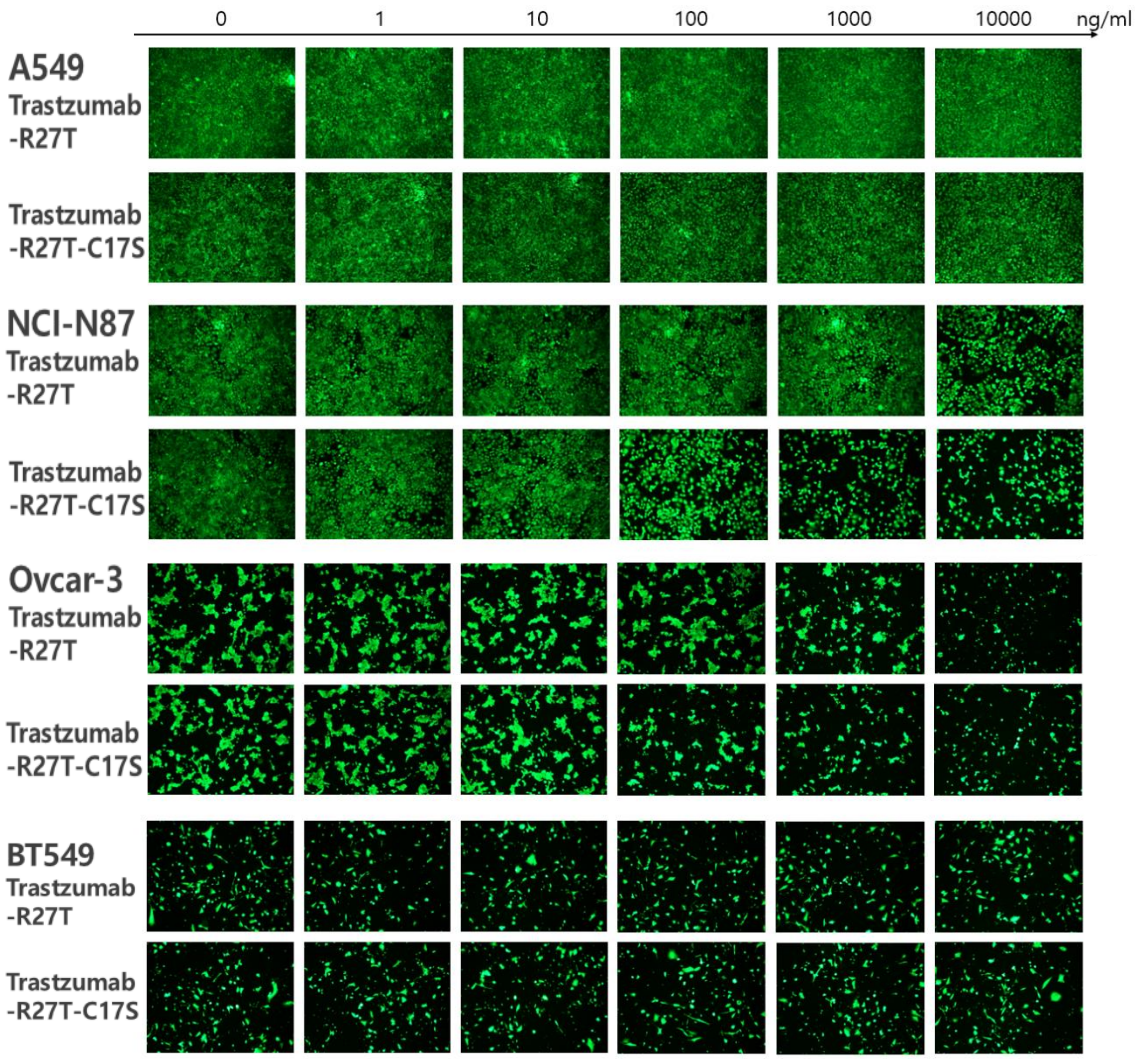


Figure 8. In vitro anti-cancer efficacy by Calcein-AM staining

Each cell was seeded in a 6-well plate and the fusion protein was treated at different concentrations and incubated for 2 days. After that, staining was performed using Calcein-AM and observed with a fluorescence microscope.

5. ADCC efficacy of Trastuzumab-C17S-R27T

induced cell-mediated cytotoxicity induced by Trastuzumab. Experiments were performed on ERBB-2 positive cells NCI-N87 and ERBB-2 negative cells BT549 and effector cells NK-92MI-CD16. In NCI-N87 cells, all three proteins showed similar efficacy. The protein treatment time was as short as 6 hours, suggesting that the immunopotentiating effect of interferon did not appear well.

In BT549, all three proteins did not show efficacy.

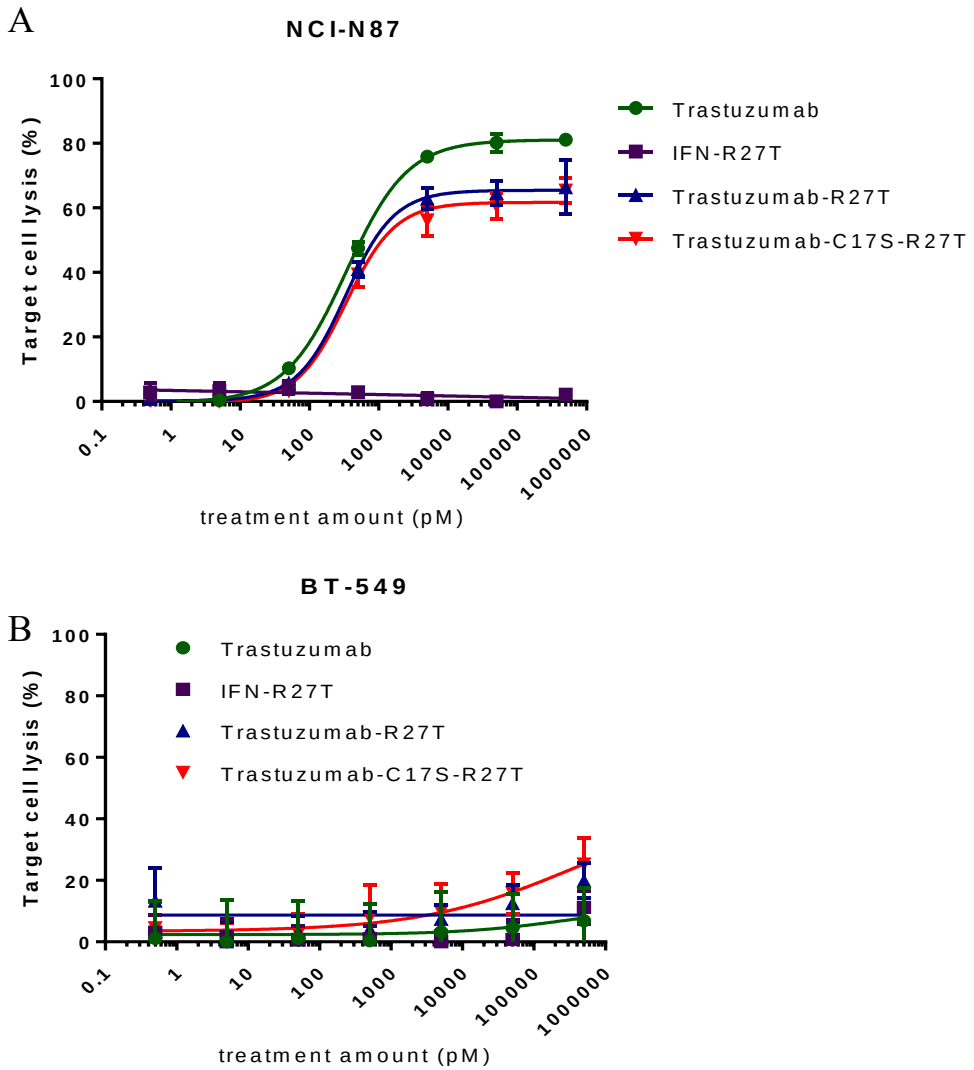


Figure 9. ***In vitro*** **ADCC efficacy of Trastuzumab-C17S-R27T**

(A) NCI-N87, ERBB-2-positive cell, was seeded 1 x 10^4 in 96-well plate and the fusion protein and effector cell NK-92MI-CD16 cells were treated and LDH assay was performed after 6 hours. (B) BT-549, ERBB-2- negative

cells, was seeded in a 96-well plate and the fusion protein and effector cell NK-92MI-CD16 cells were treated and LDH assay was performed after 6 hours.

DISCUSSION

This study compared and analyzed the properties of proteins substituted with serine for cysteine. We set two hypotheses for the effect of this substitution. The first is the improvement of physical properties. The substituted proteins showed higher purification efficiency and the active ingredient content in the affinity chromatography, and the purified products showed the same or higher efficacy at the same concentration. This is presumably due to the possibility that the free-SH groups of cysteine may interact with each other, by replacing cysteine with serine to turn the free-SH group into an -OH group to block the formation of disulfide bonds. It is considered that the formation of such a disulfide bond in the purification process may lower the yield. In fact, the purification efficiency was improved in the form of replacing cysteine with serine. However, due to the nature of interferon, the purification efficiency is lower than that of pure antibodies. Through secondary purification through ion exchange chromatography, impurities can be reduced and the purity of the protein with an effective structure can be further increased, and the second purified protein exhibits the same or higher efficacy than the first purified protein. However, there is a tendency for higher aggregation to occur due to the higher concentration of interferon mutants in the higher effective structure.

Second, it is the improvement of the efficacy by improvement of physical properties. If the individual interferon mutants in the active state are increased due

to the improvement in physical properties, a higher effect can be observed when the same concentration of the fusion protein is administered. The results of the experiments support this hypothesis. In the experiment with the effect of the antibody as the main variable, the results of administration based on the antibody concentration were similar. On the other hand, in experiments in which the effect of interferon mutants was the main variable, the efficacy of the fusion protein with the substituted interferon mutant increased significantly when administered based on the antibody concentration. This is thought to be due to the presence of more interferon with more activity. However, when analyzing the results after ion exchange purification, this tendency disappears and the two proteins show almost equal efficacy. This is because the ratio of 55 kDa to 90 kDa contained in the result of the affinity purification is different in the two proteins, but the protein having an ion exchange purification has a protein around 90 kDa.

In addition, the ADCC-confirmed experiment showed a slight decrease in the ADCC effect of the interferon-linked protein. This suggests that interferon is linked to the Fc region of the antibody and may interfere with physical binding. Therefore, ADCC can be further improved by further adjusting the length of the linker.

In a FACS experiment to observe ERBB-2 targeting, it was observed that Trastuzumab-R27T and Trastuzumab-C17S-R27T treated with ERBB-2 showed a greater shift than Trastuzumab in the low cell line. This is probably due to the interferon part of Trastuzumab-R27T and Trastuzumab-C17S-R27T interacting with the interferon receptor. In addition, Trastuzumab-R27T slightly shifted compared to

Trastuzumab-C17S-R27T, suggesting that there may be interactions between interferons in Trastuzumab-R27T.

Therefore, when the results of this experiment are applied, higher productivity and higher effect can be obtained, which may be useful in industrial aspects and economical aspects. However, the inherent toxicity problem of interferon beta is expected to require additional confirmation experiments. As the efficacy increases, the toxicity may increase as well. In order to reduce the toxicity of interferon, it is considered to form an antibody in the form of a heterodimer which binds an interferon mutant only to one side of the heavy chain of the antibody.

국문초록

인터페론 베타는 암세포의 사멸을 유도할 수 있어 항암제로 관심을 받고 있다. 그러나 인터페론 베타는 낮은 단백질 안정성과 투여 후 전신독성의 유발, 암세포의 저항성은 인터페론 베타를 항암제로 사용하는데 있어서 제한되고 있다. 이를 극복하기 위해서 항체와 융합단백질을 만드는 시도가 이루어지고 있다. 선행연구에서 본 연구그룹은 80번 아미노산에 N-glycosylation을 가지고 있는 재조합 인간 인터페론 베타1a(rhIFNβ-1a)의 27번 아미노산인 Arginine을 Threonine으로 치환하여 25번 아미노산에 추가적인 Glycosylation을 유도한 rhIFNβ-1a을 개발하였고 R27T로 명명하였으며, 이를 다시 항-ERBB-2 치료용 항체인 Trastuzumab에 융합시킨 융합단백질에 관한 개발연구를 진행하였다. 여기에 추가로 물성과 정제 효율성을 개선하기 위하여 융합단백질의 rhIFNβ-1a 부위의 17번 아미노산인 Cysteine을 Serine으로 치환한 Trastuzumab-C17S-R27T를 연구개발 하였다.

본 연구는 Protein A와 Ion exchange를 이용한 정제에서 Trastuzumab-R27T와 Trastuzumab-C17S-R27T의 정제 효율과 생물 활성을 비교 분석하였으며, 다양한 암세포주에서 Trastuzumab-R27T와 Trastuzumab-

C17S-R27T의 인터페론 생물 활성과 Immunomodulatory 효능, 항암 효능, HER2에 결합하는 능력, ADCC 효능 등을 확인하였다. Trastuzumab-C17S-R27T는 Trastuzumab-R27T와 비교하였을 때, 정제 효율이 향상되었고, 항암 효과 및 인터페론 생물 활성과 Immunomodulatory 효능에서도 동등함을 확인하였다. 추가로 HER2에 결합하는 능력, ADCC 효능들도 Trastuzumab-R27T와 동등하게 나타났다.

따라서 Cysteine을 Serine으로 치환한 Trastuzumab-C17S-R27T는 기존 Trastuzumab-R27T를 대체하여 더욱 효율적인 항암제로서의 활용 가치가 확인되었다.

주요어 : 인터페론, Trastuzumab-C17S-R27T, 면역치료, 항체치료제, 항암, 융합단백질, Trastuzumab

학 번 : 2017-22842

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